Safety and efficacy of high-dose adeno-associated virus 9 encoding sarcoplasmic reticulum Ca^{2+} adenosine triphosphatase delivered by molecular cardiac surgery with recirculating delivery in ovine ischemic cardiomyopathy

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Objective: Therapeutic safety and efficacy are the basic prerequisites for clinical gene therapy. We investigated the effect of high-dose molecular cardiac surgery with recirculating delivery (MCARD)-mediated adeno-associated virus 9 (AAV9)/sarcoplasmic reticulum Ca^{2+} adenosine triphosphatase (SERCA2a) gene delivery on clinical parameters, oxidative stress, humoral and cellular immune responses, and cardiac remodeling.

Methods: Ischemic cardiomyopathy was generated in a sheep model. The sheep were assigned to 1 of 2 groups: control (n = 10) and study (MCARD, n = 6). The control group underwent no intervention and the study group received 10^{14} genome copies of AAV9/SERCA2a 4 weeks after infarction.

Results: Our ischemic model produced reliable infarcts leading to heart failure. The baseline ejection fraction in the MCARD group was $57.6\% \pm 1.6\%$ versus $61.2\% \pm 1.9\%$ in the control group ($P > .05$). At 12 weeks after infarction, the MCARD group had superior left ventricular function compared with the control group: stroke volume index, 46.6 ± 1.8 versus 35.8 ± 2.5 mL/m² ($P < .05$); ejection fraction, $46.2\% \pm 1.9\%$ versus $38.7\% \pm 2.5\%$ ($P < .05$); and left ventricular end-systolic and end-diastolic dimensions, 41.3 ± 1.7 versus 48.2 ± 1.4 mm and 51.2 ± 1.5 versus 57.6 ± 1.7 mm, respectively ($P < .05$). The markers of oxidative stress were significantly reduced in the infarct zone in the MCARD group. No positive T-cell-mediated immune response was seen in the MCARD group at any point. Myocyte hypertrophy was also significantly attenuated in the MCARD group compared with the control group.

Conclusions: Cardiac overexpression of the SERCA2a gene by way of MCARD is a safe therapeutic intervention. It significantly improves left ventricular function, decreases markers of oxidative stress, abrogates myocyte hypertrophy, arrests remodeling, and does not induce a T-cell-mediated immune response. (J Thorac Cardiovasc Surg 2014;148:1065-73)

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Heart failure (HF) is a major public health problem, affecting >23 million people worldwide. In recent decades, improvements have occurred in myocardial revascularization and medical therapies, reducing 5-year mortality; however, the number of patients developing HF has steadily increased.¹ One explanation for these phenomena is that current surgical and therapeutic approaches are palliative and do not address the molecular pathogenesis of HF. Delivery of recombinant DNA to cardiac myocytes in situ for correction of genetically acquired transformations or by overexpression of critical signaling components that have been downregulated is an attractive proposition. It has become clear, however that gene therapy is a complex process in which tissue targeting, route of delivery, cellular trafficking, regulation of gene expression, biologic activity, dose of the therapeutic transgene, and many other factors must be considered. In our view, before the advent of molecular cardiac surgery with recirculating delivery (MCARD), systems allowing for gene delivery to most myocytes in situ did not exist. Thus, the results of the Calcium Upregulation

Abbreviations and Acronyms

AAV	= adeno-associated virus
HF	= heart failure
LV	= left ventricular
MCARD	= molecular cardiac surgery with recirculating delivery
MRI	= magnetic resonance imaging
NAb	= neutralizing antibody
ROS	= reactive oxygen species
SERCA2a	= sarcoplasmic reticulum Ca^{2+} adenosine triphosphatase
TOAC	= total antioxidant capacity

by Percutaneous Administration of Gene Therapy in Cardiac Disease trial using an inefficient intracoronary delivery route showed no demonstrable significant improvement in left ventricular (LV) function in patients in any group, using doses up to and including 10^{13} genome copies of adeno-associated virus (AAV) 1 encoding sarcoplasmic reticulum Ca^{2+} adenosine triphosphatase (SERCA2a).² Preclinical data using the same dose of AAV1/SERCA2a and the same delivery approach resulted in detectable gene expression in 0% (3 of 6) and <1% (3 of 6) myocytes in the ovine heart.³ Using simple antegrade intracoronary delivery, >99% of the vector promptly disappears into the systemic circulation.⁴ Thus, we believe that gene delivery is the rate-limiting barrier to effective gene therapy for HF.

We, and others, have shown that gene transfer with SERCA2a improves ventricular function in an experimental model of HF.^{5,6} However, SERCA2a overexpression enhances the heart's energetic state and might potentially increase the probability of ventricular arrhythmias.^{7,8} Therefore, a careful analysis of the adverse cardiac effects of SERCA2a, especially in high doses, using an efficient delivery system such as MCARD, must be completed before clinical translation.

For our study, we chose AAV, currently among the most frequently used viral vectors for gene therapy. The AAV1 vector was used in a phase I/II HF gene therapy clinical trial.² We have also used AAV1 and AAV6 in previous studies.^{5,9} To achieve prolonged cardiotropic transgene expression that will be stable for ≤ 1 year, it was shown that AAV9 might be superior to other serotypes.^{10,11} Nonetheless, AAV vectors have been shown to evoke cellular and humoral immune responses, with T cells activated toward the expressed transgene and vector capsid proteins.¹² Therefore, we hypothesized that combining a high transgene dose with a highly efficient, cardiac-specific delivery method, such as MCARD, would simultaneously enhance efficacy and limit potentially dangerous systemic exposure, abrogating an untoward immune response.

In the present study, we investigated the effect of MCARD-mediated high-dose AAV9/SERCA2a on LV function, therapeutic safety of multiple organs, oxidative stress, cellular and humoral immune responses, and cardiac remodeling in a relevant preclinical model of HF.

METHODS**Animals and Study Design**

All the sheep received humane care in compliance with the National Institutes of Health regulations, and the Carolinas Medical Center approved the protocol. An ischemic cardiomyopathy model was generated in 16 Dorsett male sheep weighing 49.7 ± 2.5 kg. The sheep were then randomly divided into 2 groups: a control group ($n = 10$) and study group ($n = 6$). After 4 weeks, the sheep in the study group underwent cardiopulmonary bypass, including MCARD-AAV9/SERCA2a gene transfer. Hemodynamic and energetic studies were conducted at baseline and 3 and 12 weeks after infarction in both groups. The sheep were then humanely killed, and the tissue was harvested and stored for additional analysis.

Animal Model of HF

The proximal portion of the first and second branches of the circumflex artery were identified and surgically ligated as previously described.⁵

MCARD and Gene Delivery

The surgical details of the MCARD procedure have been previously described.^{5,9} In brief, after sternotomy, cardiopulmonary bypass was established by way of bicaval and right carotid artery cannulation. The aorta was cross-clamped. Cold (4°C) del Nido cardioplegia solution was delivered antegrade. Closed-loop cardiac isolated circuit flow was initiated. The virus solution, consisting of 10^{14} genome copies of AAV9/SERCA2a, was injected into the retrograde coronary sinus catheter and recirculated for 20 minutes. The circuit was then flushed to wash out residual vector. After crossclamp removal, the sheep were weaned from cardiopulmonary bypass, and the chest was closed. All the sheep received critical cardiac postoperative care.

Recombinant AAV9/SERCA2a Vector Construction

Vector production, harvest, purification, and testing were done from cell lysates by the Penn Vector Core (available at: <http://www.med.upenn.edu/gtp/vectorcore/>). The recombinant AAV9.SERCA2a vector used in the present study contains an AAV serotype-9 viral capsid and a single strand: ~4.5-kb DNA containing the human SERCA2a cDNA driven by a cytomegalovirus immediate-early promoter/enhancer, a hybrid intron, and a bovine growth hormone polyadenylation signal.

Cardiac Parameter Measurements

Six-lead electrocardiography (MAC 1600; Hewlett-Packard, Palo Alto, Calif) was performed in all resting sheep at baseline, immediately after infarction, and at 12 weeks, with analysis of the waveform components. Transthoracic and transepical echocardiography with color flow Doppler was performed using a 7-MHz transducer (Acuson; Siemens Healthcare, Mountain View, Calif). Magnetic resonance imaging (MRI; Signa LX, GE Healthcare, Buckinghamshire, United Kingdom) acquisition and analysis of cardiac hemodynamics were generated for each sheep at the selected points. Evaluation of the cardiac cycles was obtained using cardiac MRI software (available at: <http://www.segment.heiberg.se>).

Polymerase Chain Reaction Detection of AAV Vector Genome

RNA and DNA were isolated from specimens using the Chemagen (Perkin-Elmer, Baesweiler, Germany) MSM1 kits to set up separate reverse transcriptase-polymerase chain reaction (PCR) and quantitative PCR

assays for SERCA2a mRNA and viral AAV9.CMV.SERCA2a DNA. To examine the expression of SERCA2a, customized kits designed by Abiomed Systems (Abiomed, Inc, Danvers, Mass) generated the SERCA2a primers. The level of ovine glyceraldehyde 3-phosphate dehydrogenase RNA was used to calculate the normalized expression using the $\Delta\Delta C_t$ method for the reverse transcriptase-PCR values. Absolute genomic copies of SERCA2a DNA were quantified using the standard normal curve.

Histologic and Electron Microscopic Assessment of Mitochondrial Abundance and Fractional Volume

The LV sections were stained with hematoxylin and eosin. Each field was scanned using a computer-generated microscale and analyzed blindly using 9 to 11 randomly selected tissue sections from each of the hearts using image analysis software. For mitochondrial assessment, the samples were analyzed using a CM 10 transmission electron microscope (Philips, Amsterdam, The Netherlands). The images were captured using a digital camera system. The mitochondrial abundance per unit area and fractional volume of mitochondria were estimated using the unbiased morphometric stereologic approach, as previously described.¹³

Oxidative Markers (Malondialdehyde, 4-Hydroxynonenal)

LV tissue was homogenized and centrifuged (Fisher Scientific, ThermoFisher Scientific, Atlanta, Ga). The protein concentration was measured using a protein assay kit according to the manufacturer's protocol (Bio-Rad Laboratories, Inc, Hercules, Calif). Protein conjugates of malondialdehyde and 4-hydroxynonenal were measured using enzyme immunoassays (Cell Biolabs, Inc, San Diego, Calif).

Total Antioxidant Capacity

The total antioxidant capacity (TAOC) was measured in LV samples using a colorimetric microplate assay for total antioxidant power (Oxford Biomedical Research, Rochester Hills, Mich). TAOC was expressed in Cu^{2+} -reducing equivalents against a uric acid standard and normalized for the protein content of the sample.

T-Cell-Mediated Response (Interferon- γ Enzyme-Linked ImmunoSpot Assay)

Interferon- γ Enzyme-Linked ImmunoSpot assays (Mabtech, Inc, Cincinnati, Ohio) were performed according to a previously described protocol.¹⁴ Peptide-specific cells were represented as spot-forming cells per 10^6 cells and were calculated by subtracting the spot numbers.

AAV9 Neutralizing Antibody Assay

Neutralizing antibody (NAb) assays were performed by incubating the serotype 9 of AAV.CMV.LacZ with twofold dilutions of heat-inactivated sheep serum and determining the β -galactosidase expression in Huh7 cells. The limit of detection for the assay is 1:5 serum dilution. AAV9-specific total NAb titers were determined using an enzyme-linked immunosorbent assay.¹⁴

Statistical Analysis

All data are presented as the average $\pm$ standard error of the mean. The Kolmogorov-Smirnov test for normality was applied to each data set. Single analysis of variance was performed across time (eg, baseline, 3 weeks, 12 weeks) for each parameter. Next, unpaired *t* tests were used to assess the differences between any 2 points or differences at the same time between the 2 groups (eg, study vs control).

RESULTS

Model of Ovine Postischemic HF and Operative Results

All the sheep in both groups sustained a large transmural posterolateral infarct with moderate to severe LV

FIGURE 1. A, Quantitative polymerase chain reaction of sarcoplasmic reticulum Ca^{2+} adenosine triphosphatase (SERCA2a) biodistribution in heart and liver tissue (genome copies/100 ng DNA). B, Relative expression by reverse transcriptase-quantitative polymerase chain reaction of SERCA2a/glyceraldehyde 3-phosphate dehydrogenase in heart and liver tissue. Gray columns, molecular cardiac surgery with recirculating delivery adeno-associated virus 9/SERCA2a group; black columns, control group. **P* = .05 for differences between groups; ‡*P* < .05 for differences within groups. AAV, Adeno-associated virus; LV, left ventricular; mRNA, messenger RNA.

dysfunction. In the study group (MCARD-AAV9/SERCA2a), all sheep survived without incident and were humanely killed at 12 weeks after infarction. The average cardiopulmonary bypass and crossclamp time was 143 ± 6 and 77 ± 5 minutes, respectively. In 1 case in the control group, the sheep developed refractory ventricular fibrillation after coronary ligation and was excluded from the study. The LV infarct size was similar between the control and study groups ($19.4\% \pm 2.1\%$ vs $20.6\% \pm 1.6\%$; *P* > .05).

Cardiac and Liver Expression After High-Dose AAV9/SERCA2a Gene Transfer

The cardiac tissue delivery of SERCA2a genome copies in the MCARD-AAV9/SERCA2a group was significantly increased in the LV anterior and lateral walls compared with the liver and heart tissue from the control group (*P* < .05 for both; Figure 1, A). In contrast, no difference was seen in the SERCA2a genome copies in the liver between the 2 groups. The same trends were observed using reverse transcriptase-quantitative PCR to assess the relative expression of SERCA2a in the cardiac samples in the study group compared with the control group (Figure 1, B).

Cardiac Function and LV Remodeling

Global cardiac function was not significantly different between the 2 groups on the baseline MRI scan. Creation of myocardial infarction resulted in deterioration of heart

FIGURE 2. Baseline and 3- and 12-week postinfarction values for (A) left ventricular (LV) ejection fraction (%), (B) stroke volume index (mL/m²), (C) end-systolic diameter (mm), (D) end-diastolic diameter (mm), (E) left ventricular wall thickness (mm), and (F) left ventricular fractional wall thickening (%). Gray columns, molecular cardiac surgery with recirculating delivery of adeno-associated virus 9/sarcoplasmic reticulum Ca²⁺ adenosine triphosphatase group; black columns, control group. * $P < .05$ for differences between groups.

performance in both groups. At 3 weeks in the MCARD and control groups, a decrease in ejection fraction by $22.6\% \pm 5.5\%$ and $24.7\% \pm 3.4\%$ and an increase in end-diastolic volume by $22.7\% \pm 6.0\%$ and $22.0\% \pm 6.7\%$ and end-systolic volume by $57.8\% \pm 6.2\%$ and $71.0\% \pm 11.4\%$, respectively, was seen ($P > .05$ for all). At 12 weeks, the MCARD-AAV9/SERCA2a-treated HF sheep showed significant improvement in systolic and diastolic LV function compared with the control group (ejection fraction, $46.2\% \pm 1.9\%$ vs $38.7\% \pm 2.5\%$; stroke volume index, 46.6 ± 1.8 vs 35.8 ± 2.5 mL/m²; end-systolic diameter, 41.3 ± 1.7 vs 48.2 ± 1.4 mm; end-systolic volume, 66.7 ± 5.0 vs 80.3 ± 5.1 mL; end-diastolic diameter, 51.2 ± 1.5 vs 57.6 ± 1.7 mm; $P < .05$ for all). Analysis of LV remodeling revealed significantly decreased LV end-systolic volumes and end-diastolic dimensions after gene therapy (Figure 2, A-D).

In line with improvements in global cardiac function, the assessment of regional contractility demonstrated that the LV wall thickness in the MCARD group was relatively stable and remained approximately 11 mm across the follow-up points. This finding suggested a high likelihood of recovery of regional contractile function after myocardial infarction.¹⁵ In contrast, in the control group, the LV

thickness had decreased significantly after 12 weeks and the chances of recovery were very low (Figure 2, E). Also, LV fractional systolic wall thickening was significantly greater in the MCARD-AAV9/SERCA2a group at 12 weeks than in the control group ($48.3\% \pm 2.9\%$ vs $17.6\% \pm 3.7\%$, $P < .01$; Figure 2, F).

Myocardial Hypertrophy, Mitochondrial Abundance, and Fractional Volume

The cross-sectional areas of myocytes from the MCARD sheep were significantly smaller than those in the control sheep (98 ± 0.7 vs 89 ± 0.7 μm^2 ; $P < .01$; Figure 3, A-C), indicating significantly less myocyte hypertrophy. The mitochondria in the control group occupied a smaller fraction of the total tissue volume than in the MCARD group ($22.0\% \pm 1.7$ vs $30.2\% \pm 1.9\%$; $P < .05$) and also exhibited a lower mitochondrial density (0.16 ± 0.01 vs 0.45 ± 0.04 per μm^2 ; $P < .05$). These findings were consistent with an increase in mitochondrial swelling in the control group (Figure 3, D-G).

Oxidative Stress and TAOC Markers

In the MCARD group, malondialdehyde and 4-hydroxynonenal conjugate concentrations were significantly

FIGURE 3. Morphometric analysis of myocytes and mitochondria. Hematoxylin and eosin-stained infarct border zone tissue cross-section (scale bar = 40 μm) from (A) the control group and (B) molecular cardiac surgery with recirculating delivery of adeno-associated virus 9/sarcoplasmic reticulum Ca^{2+} adenosine triphosphatase group (MCARD-AAV9/SERCA2a). C, Average cross-sectional cardiac myocyte area in infarct border zone (μm^2). Transmission electron microscopy of infarct border zone tissue (scale bar = 1 μm) from (D) control group (arrows indicate mitochondrial swelling) and (E) MCARD-AAV9/SERCA2a group (arrows indicate normal-appearing mitochondria). Average stereologic estimates of (F) mitochondrial fractional volume (%) and (G) mitochondrial density (per μm^2). Gray columns, MCARD-AAV9/SERCA2a group; black columns, control group. * $P < .05$ for differences between groups.

suppressed in the infarct zone compared with those in the remote zone (malondialdehyde, 0.00 ± 0.00 vs 1.51 ± 0.24 pmol/mg protein; 4-hydroxynonenal, 1.04 ± 0.41 vs 8.22 ± 0.96 $\mu\text{g}/\mu\text{g}$ protein; $P < .05$). In contrast, the control group showed no significant differences between these zones (Figure 4, A and B). We did not find any significant differences in antioxidant capacity (TAOC) between the 2 groups or within the groups ($P > .05$).

Blood Work and Electrophysiologic Safety Profile

Complete blood counts and chemistry analysis for comprehensive metabolic evaluation of all major organs and physiologic systems were conducted preoperatively and postoperatively at 12 weeks after infarction. All data are summarized in Table E1. The mean post-MCARD-AAV9/SERCA2a serologic values at 12 weeks were generally within normal limits. In particular, the levels of

creatinine, blood urea nitrogen, alanine aminotransferase, alkaline phosphatase, and total bilirubin remained well within the reference ranges.

The electrocardiographic assessment at 12 weeks after infarct showed that the QRS interval was significantly lengthened at 12 weeks in the control group. In contrast, the MCARD group had maintained baseline levels (60 ± 15 vs 34 ± 4 ms; $P < .05$). The PQ interval, QT interval, and heart rate were unaffected by treatment. As expected, the ST segments were significantly elevated after infarct creation and at 12 weeks remained slightly perturbed (Table E2). No arrhythmias were detected in either group.

Immune Response to AAV9 and SERCA2a Transgene

The T-cell immune response to AAV9 and SERCA2a transgene was evaluated using the interferon- γ Enzyme-Linked ImmunoSpot assay at selected follow-up points. None of

FIGURE 4. Oxidative markers in infarct and remote tissue zones. A, Malondialdehyde (MDA) concentration (pmol/mg protein). B, 4-Hydroxynonenal–protein conjugates in hydroxynonenal–bovine serum albumin equivalents ($\mu\text{g}/\mu\text{g}$ protein). Gray columns, molecular cardiac surgery with recirculating delivery of adeno-associated virus 9/sarcoplasmic reticulum Ca^{2+} adenosine triphosphatase group; black columns, control group. * $P < .05$ for differences within groups. BSAeq, Body surface area equivalents; HNE, 4-hydroxynonenal conjugate.

the MCARD-AAV9/SERCA2a sheep had evidence of a T-cell-mediated immune response to AAV9 capsid or human SERCA2a at any of the tested points in all sheep (Figure 5).

The presence of pre-existing antibodies is an important barrier to gene therapy, because it strongly affects transgene expression. Therefore, the sheep were prescreened for the presence of NABs against the administered AAV9. The AAV9 NAB levels at baseline in sheep who had not previously received AAV9 vector were nondetectable using an enzyme-linked immunosorbent assay; thus, all showed corresponding titers $<1:5$. The 6 MCARD sheep that received rAAV9 vector had increases in the NAB titers of >1 in 120 against AAV9 at 9 and 12 weeks after the MCARD procedure.

DISCUSSION

The consistent coronary arterial anatomy in sheep, similar to that of humans, and a lack of collateral vessels provides substantial advantages for creation of a clinically relevant ischemic cardiomyopathy model with a predictable myocardial infarct size in this species.¹⁶ This physiologically appropriate large animal model, involving ligation of the circumflex artery's first 2 branches, is accompanied by almost 0% mortality and is highly suitable for demonstrating ventricular remodeling after gene therapy.

FIGURE 5. Time course of transgene-specific T-cell responses to pools of adeno-associated virus 9 (AAV9) capsid peptides and pools of sarcoplasmic reticulum Ca^{2+} adenosine triphosphatase (SERCA2a) transgene in peripheral blood mononuclear cells (PBMCs) after administration of AAV9/SERCA2a. SFU, Number of interferon- γ spot-forming units in Enzyme-Linked ImmunoSpot assay of left ventricular tissue samples; No stim, negative control; PMA+ION, positive control. Red line indicates positive result threshold; A, B, C, and D each refer to a specific sheep in the molecular cardiac surgery with recirculating delivery-AAV9/SERCA2a group.

In the present study, we confirmed our previous efficacy claims by showing that MCARD delivery of AAV9/SERCA2a results in robust expression in the lateral and anterior walls of the left ventricle with an almost 100-fold greater level of expression than in the liver. MRI analysis showed the favorable effects of SERCA2a expression through improved global and regional cardiac mechanics and the substantial arrest of remodeling.

Postinfarction remodeling is usually divided into early and late phases. The early phase involves expansion of the infarct zone. Late remodeling is caused in part by failure to normalize the increases in wall stress and is associated with time-dependent progressive LV dilatation, distortion of the ventricular shape, deterioration of contractile function, and myocyte hypertrophy. Alterations to myocyte architecture will initially stabilize the distending forces and prevent additional ventricular deformation but are associated with increased energy consumption. Studies have indicated that in the healthy heart, the mitochondrial function and structure will match the maximal energy demand. However, in a failing myocardium, the energy and demand are mismatched.¹⁷ As expected, we found, through histologic analysis, that a loss of mitochondrial density occurred in the control group but that MCARD/SERCA2a treatment prevented mitochondrial loss, thereby preserving the high aerobic capacity of the muscle tissue. The decrease in mitochondrial density was proportionally greater than the decrease in fractional volume, suggesting evidence of maladaptive mitochondrial swelling in the control group. Morphometric analysis of the electron microscopic images in our study showed that the number of mitochondria per myocyte decreased and the volume of individual mitochondria increased in the controls but not in the treatment group. A reduction in the number of

mitochondria can probably be explained by the activation of pro-apoptotic pathways in HF, and the relative increase in mitochondrial volume (swelling) is a failed compensatory response.

It is accepted that oxidative stress is caused by an imbalance between the generation of reactive oxygen species (ROS) and antioxidant defense mechanisms and is an important regulator of cardiac remodeling.¹⁸ In contrast to the direct actions on cellular injury, ROS also play an important role in cell protection by activation and expression of antioxidant enzymes.¹⁹ Among the biologic processes, the level of lipid peroxidation is widely used as a biomarker of oxidative stress because the lipids are most susceptible to ROS. Myocytes can accumulate ROS as a result of either increased numbers of total mitochondria producing ROS or the production of higher levels of ROS by damaged mitochondria.²⁰ This can lead to the deterioration of cellular function and viability.

Natural AAV infections or AAV gene therapy can cause the development of AAV NABs in animal species and humans. Pre-existing antibodies can modulate the efficacy of gene therapy by blocking vector transduction or redirecting distribution of the AAV vectors.²¹ One of the most important aspects of the development of AAV for clinical gene therapy is the effect of the host humoral immune response against its capsid. No pre-existing AAV9 antibodies were found in any sheep in either group; however, moderate titers were found in the treatment group after gene therapy. One strategy to overcome AAV NABs in AAV-mediated gene therapy includes immunosuppression. It was demonstrated in a dog model that after LV intramyocardial injection of low-dose 10^{11} genome copies of AAV6/SERCA2a, all the dogs developed positive AAV-NAB titers >1 in 120. Immunosuppression beginning 4 weeks after AAV injection reduced the NAB titers to baseline levels.²² However, our finding of positive NAB titers 9 to 12 weeks after gene delivery was not unexpected and would not affect efficacy, given that this was single-dose therapy with no need for re-administration.

The immune response with activation of T cells directed against the viral capsid constitutes an important safety concern in the use of AAV vectors. This response is dose dependent, and delivery of 10^{12} of 10^{13} viral genomes (less than in our study) was associated with specific T-cells directed against the vector capsid.²³ Also, it is thought that capsid-directed T-cell-mediated cellular destruction is implicated in the observed toxicity in the high-dose group in the AAV2 clinical trial for the treatment of hemophilia.²⁴ In our study, we found no positive T-cell immune response in any sheep at any measurement point. This protective effect of MCARD likely resulted from the closed-loop recirculation technique used to deliver the vector in a highly organ-specific fashion, minimizing the exposure of antigen-presenting cells to capsid antigens, an

important and unique safety feature of the MCARD delivery platform.

The results of the present study have demonstrated that MCARD delivery of a high-dose of AAV9/SERCA2a is safe and efficacious, with no toxic effects on the major organ systems, no T-cell-mediated immune response, no increase in the prevalence of arrhythmias, decreased myocardial hypertrophy, and improved mitochondrial ultrastructure, density, and function. Finally, MCARD-mediated delivery of AAV9 expressing SERCA2a led to markedly improved global LV function and significantly arrested maladaptive ventricular remodeling.

Study Limitations

The present study used a relatively small number of sheep owing to the high cost and time demands of the study. A period of follow-up >12 weeks would be desirable. We did not incorporate a control group with intracoronary delivery or an MCARD-null vector and other AAV serotypes because of the high time and material costs. Furthermore, studies using AAV1 and AAV6 have been previously published,⁵ and the inefficiency of intracoronary delivery—resulting in sequestration of $>99\%$ of the injected vector in the liver—is well documented.^{3,4}

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Discussion

Dr Todd K. Rosengart (Houston, Tex). Congratulations, Dr Bridges, again, on very nice work and thank you for allowing me to review your report in advance.

As you rightly note, and I would reiterate, there is a lack of appreciation that many experimental and clinical cardiac gene therapy trials have been encumbered by inadequate substrate delivery, just as we have heard in the earlier presentations, and again we applaud you for your diligent effort in pursuing this.

The MCARD technique, I think, remains limited in the sense that you do need cardiopulmonary bypass. Also, I am sure you have looked very diligently at this, but the question is: Is there any way to accomplish the MCARD technique without actually instituting cardiopulmonary bypass, such as through a blood recirculation technique?

In regard to your data in the current technique, my second question, which was in your report, is even though you have effectively shown much higher levels of cardiac uptake compared to the liver, you did still have NABs in your controls. Do you have any further plans to improve isolation or address this issue?

Finally, recognizing that time did not allow your addressing this issue, we know the retrograde perfusion technique preferentially delivers to the left ventricle and bypasses the right ventricle and septum. Your expression with MCARD, I believe, has been heterogeneous because of this. Are there any approaches that you could add to your technique to improve right ventricular and septal delivery?

Again, congratulations on this very nice work.

Dr Bridges. Well, thanks, Todd, it is certainly a pleasure and an honor to have our report reviewed by one of the pioneers in cardiac gene therapy. Dr Rosengart conducted the first clinical trial of gene therapy using cardiac surgery as a delivery platform more than a decade ago. So, thank you for the opportunity to discuss this with you.

Your first question was: Molecular cardiac surgery with recirculating delivery, can it be done without cardiopulmonary bypass? And that has been tried. I published a letter to the editor in *Gene Therapy* in 2009 in which a “recirculating” delivery technique known as “V-Focus” was reported that purported to isolate the heart in situ. In fact, they actually did use a perfusion circuit, so even that was not without cardiopulmonary bypass.

However, owing to the complexities of the cardiac circulation with the Thebesian veins, for example, and in the sheep model with the hemizygous vein, it is really difficult, if not impossible, to effectively isolate the heart without cardiopulmonary bypass, at least at present. Of course, that remains an open question. We have developed some technology that should allow us to do it minimally invasively, but at present, to achieve the 99% efficacious separation of the 2 circuits requires cardiopulmonary bypass. However, we are certainly interested in pursuing other options, which might not be perfect but would enhance the effectiveness of catheter-based delivery.

Your second question was the NABs. We did, in fact, see evidence of NABs in our experimental group despite our 99% efficient separation of the cardiac from the systemic circulation. We published a report in the *Journal of Cardiac Failure* in 2011, which was a pharmacokinetic analysis. The concern is patients who have NABs before you administer the gene therapy, which decreases the efficacy of the gene delivery. If a patient develops NABs after gene delivery, it might or might not have an effect on subsequent treatment but will not affect the efficacy of the therapy. However, with most of these gene therapy protocols, one-time treatment is really what we expect is likely to be efficacious, so hopefully that will not be an important limitation. Apparently, even the small amounts of antibody of the viral vector that moved into the circulation with our technique, which, again, was orders of magnitude less than with the intracoronary approach, was enough to provoke a NAB.

Finally, your question was about retrograde versus antegrade. As you know, retrograde delivery more effectively transduces or provides nutrient flow using retrograde cardioplegia to the left ventricle and is less efficient in the right ventricle; and, in fact, that is what we have seen in our work, in our previously published work, as recently as a report published in August 2013 in the *Annals of Thoracic Surgery*. However, although we have seen bet-

ter transduction of the left ventricle than the right ventricle, we have seen at least 10 to 100 times better transduction of even the right ventricle using this technique than with the intracoronary delivery method. Thus, hopefully, that will still be enough to achieve efficacy on the right side. Also, you could combine retrograde with antegrade. Thus, a number of potential strategies exist that might allow us to overcome that.

TABLE E1. Complete blood count and blood chemistry summary

Variable	Reference range	Baseline	12-wk point
Complete blood count			
Hemoglobin (g/dL)	9-15	12.2 ± 0.5	8.4 ± 0.6
Total WBC ($10^3/\mu\text{L}$)	4-12	9.0 ± 0.7	9.0 ± 0.7
Total RBC ($10^6/\mu\text{L}$)	9.0-15.8	11.4 ± 0.4	8.4 ± 0.6
Hematocrit (%)	27-45	33.0 ± 1.2	24.7 ± 1.5
Platelet count ($10^3/\mu\text{L}$)	100-800	430 ± 71	415 ± 61
Neutrophils (%)	10-50	30.8 ± 2.3	35.2 ± 4.7
Lymphocytes (%)	40-75	64.2 ± 3.1	63.8 ± 4.9
Renal system			
Urea nitrogen (mg/dL)	10-35	14.0 ± 1.2	13.4 ± 1.7
Sodium (mEq/L)	145-152	146.5 ± 1.1	141.4 ± 0.8
Potassium (mEq/L)	3.4-5.5	5.5 ± 0.3	4.7 ± 0.1
Creatinine (mg/dL)	0.2-1.9	0.70 ± 0.06	0.64 ± 0.08
Gastrointestinal system			
Liver function			
Alanine aminotransferase (U/L)	22-38	16.2 ± 1.4	29.0 ± 5.2
Alkaline phosphatase (U/L)	70-390	180 ± 27	76 ± 11
Total bilirubin (mg/dL)	0.1-0.5	0.18 ± 0.03	0.34 ± 0.05
Albumin (g/dL)	2.4-4.0	3.17 ± 0.08	2.21 ± 0.15
Pancreatic function			
Lipase (U/L)	5-35	15.0 ± 2.3	10.0 ± 8.0
Amylase (U/L)	22-35	13.2 ± 2.5	10.7 ± 1.4
Glucose (mg/dL)	50-80	62.3 ± 3.5	74.0 ± 3.1
Complete blood count			
Hemoglobin (g/L)	90-150	122 ± 5	84 ± 6
Total WBC ($10^9/\text{L}$)	4-12	9.0 ± 0.7	9.0 ± 0.7
Total RBC ($10^{12}/\text{L}$)	9.0-15.8	11.4 ± 0.4	8.4 ± 0.6
Hematocrit	0.27-0.45	0.33 ± 0.01	0.25 ± 0.01
Platelet count ($10^9/\text{L}$)	100-800	430 ± 71	415 ± 61
Neutrophils ($10^9/\text{L}$)	0.7-6.0	4.0 ± 0.5	2.8 ± 0.3
Lymphocytes ($10^9/\text{L}$)	2-9	4.5 ± 0.6	6.1 ± 0.6
Renal system			
Urea nitrogen (mmol/L)	3.6-12.5	5.0 ± 0.4	4.8 ± 0.6
Creatinine ($\mu\text{mol}/\text{L}$)	18-168	62 ± 5	56 ± 7
Sodium (mmol/L)	145-152	146.5 ± 1.1	141.4 ± 0.8
Potassium (mmol/L)	3.4-5.5	5.5 ± 0.3	4.7 ± 0.1
Gastrointestinal system			
Liver function			
Alanine aminotransferase (U/L)	22-38	16.2 ± 1.4	29.0 ± 5.2
Alkaline phosphatase (U/L)	70-390	180 ± 27	76 ± 11
Total bilirubin ($\mu\text{mol}/\text{L}$)	1.7-8.6	3.2 ± 0.5	5.8 ± 0.8
Albumin (g/L)	24-40	31.7 ± 0.8	22.1 ± 1.5
Pancreatic function			
Lipase (U/L)	5-35	15.0 ± 2.3	10.0 ± 8.0
Amylase (U/L)	22-35	13.2 ± 2.5	10.7 ± 1.4
Glucose (mmol/L)	2.8-4.4	3.5 ± 0.2	4.1 ± 0.2

WBC, White blood cell; RBC, red blood cell.

TABLE E2. Electrocardiographic segment analysis summary

ECG wave interval	Baseline	After acute MI	12-wk follow-up point	
			MCARD-SERCA2a	Control (no treatment)
PQ interval (ms)	124 ± 8	136 ± 10	133 ± 17	126 ± 16
QRS interval (ms)	32 ± 4	21 ± 7	34 ± 4	60 ± 15
QT interval (ms)	305 ± 23	343 ± 25	339 ± 37	348 ± 17
ST deviation (μV)	−37.5 ± 14	346 ± 80	33 ± 17	27 ± 10
Heart rate (beats/min)	113 ± 13	103 ± 17	100 ± 16	96 ± 7

ECG, Electrocardiographic; MI, myocardial infarction; MCARD-SERCA2a, molecular cardiac surgery with recirculating delivery of sarcoplasmic reticulum Ca²⁺ adenosine triphosphatase.